Higher levels of CD19+ leukocytes in Gaucher disease patients as a potential marker for malignancy

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Abstract

Aim: Gaucher disease is an autosomal recessive lysosomal storage disease caused by insufficient glucocerebrosidase activity resulting in accumulation of glucosylceramide, particularly in macrophages. Multiple myeloma and B cell lymphoma are considered to be one of the causes of death from GD in the long term. We aimed to compare cell surface antigens of leukocytes to try to identify a reliable marker for leukocyte infiltration and progression to lymphoid malignancy. Material ve Method: 10 Gaucher disease patients and 20 age-matched healthy controls were included. Leukocytes were collected from whole blood using a Ficoll gradient, stained for specific cell surface antigens (CD33, CD19, CD14, and CD8) and sorted by flow cytometry. Levels of each leukocyte cell surface antigen were expressed as a percentage of leukocytes expressing them. Leukocyte glucocerebrosidase activity was measured by fluorometry. Results: The percentage of CD19+ leukocytes in Gaucher disease patients (8.2 ± 3.4) was significantly higher than in the control group (4.8 ± 3.4) ($p<0.05$). The percentage of leukocytes expressing CD33 (12.8 ± 6.6 vs 7.9 ± 8.0, p=0.077), CD14 (10.6 ± 4.6 vs 7.1 ± 6.9, p=0.094) or CD8 (12.7 ± 5.3 vs 9.8 ± 5.9, p=0.115) was not significantly higher in patients than in controls. Discussion: The higher levels of CD19+ leukocytes may serve as a useful marker to predict leukocyte infiltration and perhaps also malignancy in Gaucher disease patients. Experimental anti-CD19 drugs are in development for the treatment of B cell cancers, and CD19+ leukocyte levels may also serve as a marker of the response to this treatment.

Keywords

Gaucher Disease; Leucocyte Cell Surface Antigens; CD19+; Multiple Myeloma; Leucocyte Infiltration; Flow Cytometry

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Introduction

Gaucher disease (GD) is an autosomal recessive lysosomal storage disease caused by insufficient glucocerebrosidase activity resulting in cellular accumulation of glucosylceramide, particularly in leukocytes and of them especially in macrophages. The patients manifest a wide variety of signs from delayed growth to neurologic symptoms. Thus, the disease can be classified as non-neuronopathic (Type 1) or neuronopathic (Types 2 and 3). The majority (95% of cases) is Type 1 GD patients with splenomegaly, hepatomegaly, anemia, thrombocytopenia, bone disease and delayed growth. Type 2 is the fatal form that is characterized by a fast brainstem degeneration and irresponsiveness to treatment. Type 3 manifests with neurologic symptoms and limited lifespan (<30 years) [1]. The current treatment of GD is based on enzyme replacement therapy (ERT), imiglucerase and follow-up studies show that the response is maintained only when the ERT is administered every two weeks [2].

Lysosomal accumulation of b-glucosylceramide and its deacylated product, glucosylsphingosine is associated with chronic inflammation and B-cell activation, often manifested by polyclonal and monoclonal gammopathy [3,4]. Several possible contributing factors like gene modifiers, splenectomy and immune system deregulation induced by cytokines, chemokines, and hydrolases released from Gaucher cells have also been proposed for increased B-cell proliferation and neoplasia in GD. A greater incidence of B cell non-Hodgkin lymphoma and some other hematologic malignancies (multiple myeloma, plasma cell malignancies, hepatocellular carcinoma) in GD patients has recently been reported [5] and the triggering role of bioactive sphingolipids on the known clonal expansion of immunoglobulin-secreting B cells and the pathogenesis of multiple myeloma has been investigated [6,7]. Multiple myeloma and B cell lymphoma are suspected to be the leading causes of death in GD patients over the long term [5,8].

B-lymphocyte antigen CD19 is a glycoprotein expressed on B cells and is present from the earliest recognizable B-lineage cells during development to B cell blasts, but is lost on maturation to plasma cells. Since CD19 is a hallmark of B cells, the protein has been used to diagnose cancers that arise from this type of cell, notably B cell lymphomas [9]. Other related antigens include: CD33, which is usually considered to be a myeloid-specific transmembrane receptor expressed on cells of myeloid lineage; CD14, which serves as a co-receptor for the detection of bacterial lipopolysaccharide; and CD8, which is a transmembrane glycoprotein predominantly expressed on the surface of cytotoxic T cells and serves as a co-receptor for the T cell receptor.

In this preliminary study, our aim was to compare the expression of leukocyte cell surface antigens in GD patients and healthy controls and glicocerebrosidase enzyme activity in the specific leucocytes sorted by flow cytometry, in order to investigate possible relationship between the disease severity and leucocyte antigens in the hope of finding a reliable marker for leukocyte infiltration and progression to lymphoid malignancy in GD.

Material and Method

Patients

Ten patients with genetically verified GD Type 1 and Type 3 (3 males and 7 females, 3–40 years of age) and 20 age-matched healthy controls (8 males and 12 females) were included in this study. The protocol was approved by the Ethical Committee of the Ege University School of Medicine (13-3.1/4, 19.03.2013). Informed consent was obtained from all participants and the legal guardians of the children included in the study.

Sample collection and processing

We collected 8 mL of whole blood from patients just before they received enzyme replacement therapy and from controls. Blood was anticoagulated with EDTA. Leukocytes were separated manually using a Ficoll gradient.

Flow cytometry

The leukocytes were labelled with different dye-labelled antibodies specific for each of four cell surface antigens (CD14, CD8, CD33 and CD19) as follows: CD14 was stained with peridinin chlorophyll protein (PerCP)-Cy5-5-labeled anti-CD14; CD8 with phycoerythrin (PE)Cy7-A-labeled anti-CD8; CD33 with fluorescein isothiocyanate (FITC)-A-labeled anti-CD33 and CD19 with PE-A-labeled anti-CD19 (all labelled antibodies were purchased from BD Biosciences). The cells were sorted by flow cytometry, and the leukocytes positive for CD33, CD19, CD14, and CD8 were collected. Figure.1 presents the distribution of leukocytes positive for signed antigens.

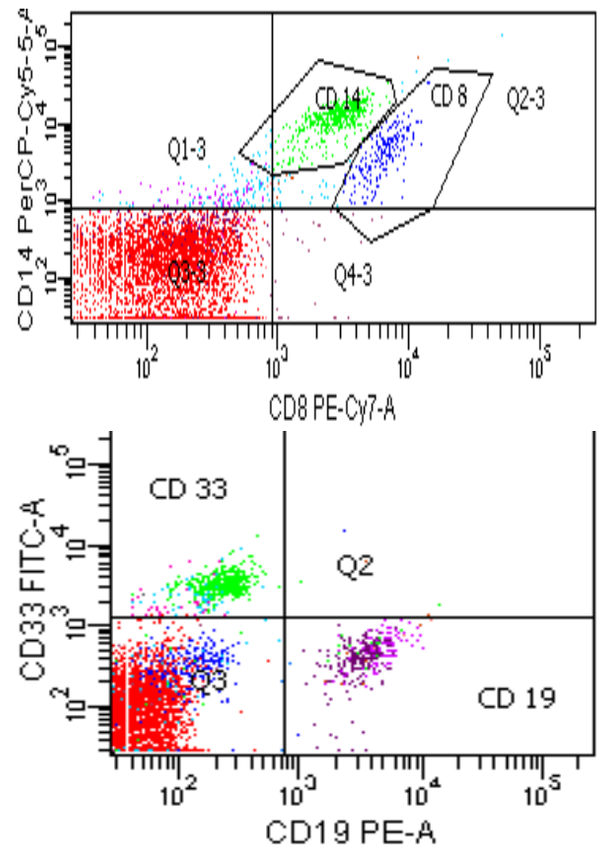

Figure.1. The graphical presentation of signed antigens in Flow cytometry. For clear discrimination of antigen presenting leukocytes, CD19 PE-A is paired with CD33FITC-A, and CD8 PE-CyA is paired CD14PerCP-Cy5-5.

Measurement of glucocerebrosidase activity

Glucocerebrosidase enzyme activity (nmol/h/mg protein) was determined fluorometrically in the leukocyte samples after sorting by the method of Chamoles *et al.* [10] with sample blank correction [11]. Briefly, 5 µl of leukocyte for each measurement were placed in a 96-well microplate. After the addition of an elution buffer (Na-taurodeoxycholate) and substrate (4-Methylumbelliferyl -β-D-glucopyranoside, 20 M, pH=4.4) to the well, the samples were incubated for 1h at 37oC. The reactions were stopped by adding 200 microliters of 0.1M ethylenediamine. All readings (excitation 365nm and emission 450nm) were performed on a Modulus microplate fluorometer (Turner Biosystems), and all calculations were performed using a calibration curve of 4-methylumbelliferone as the calibrator. Leukocyte samples from a healthy person and a confirmed patient (randomly selected) were used as positive and negative controls in each assay run. The protein levels of the leukocytes were determined by the Lowry method [12].

Statistical analysis

All data were evaluated using the SPSS 17.0 statistics program. Mann-Whitney U test was used to compare mean enzyme activity between the groups. Correlation analysis was performed using Spearman's correlation test.

Results

Eight of 10 patients were under enzyme replacement therapy (mean treatment duration was 6.1± 3.3 years) and 2 of them were newly diagnosed. Five patients had hepatomegaly, and 9 patients had splenomegaly, none of them have neurologic symptom/signs. The routine biochemical parameters (glucose, SGOT, SGPT, ALP, LDH, phosphorus) of patients were in the normal levels; serum calcium levels were in lower limits (8.6 ± 3.0, reference interval 8.6-10.2). The Z-score (L1-4) of patients were low (-0.63 ± 0.38) indicated secondary osteoporosis.

There was no statistically significant difference between the ages of patients in the GD group (22.2 ± 13.2) and control group (27.3 ± 3.2) (p>0.05). The leucocyte glucocerebrosidase activity of patients were lower than those of controls (1327±1448 nmol/mg protein vs. 372±465 nmol/mg protein).

Comparison of levels of each leukocyte cell surface antigen between GD patients and healthy controls is expressed as a percentage (Table 1). We found that the percentage of leukocytes bearing the CD33, CD14 and CD8 cell surface antigens were higher in GD patients than in the control group but the difference was not statistically significant. Only the percentage of CD19-positive (CD19+) leukocytes was significantly higher in GD patients than in the control group (p<0.05). We determined significantly positive correlations between the percentage of different leucocytes in controls, no correlation determined in the patient group.

The glucocerebrosidase activities in the sorted leukocytes positive for CD33, CD19, CD14, and CD8 of GD patients were lower than those of controls**.** While the enzyme activity in the different type of leucocytes (CD33+, CD19+, and CD14+) of healthy controls positively correlated with each other (Table.2), there was no correlation in patient's leucocytes.

Table 1. The percentage of leukocytes labelled with specific antibodies in patients and controls. Data are given as mean ± SD. The comparisons were made versus control (Mann Whitney u test).

	Healthy control N=20	Gaucher Disease patients N=10	significance
CD33+	7.9 ± 8.0	12.8 ± 6.6	p=0.077
CD19+	4.8 ± 3.4	8.2 ± 3.4	p=0.024
CD14+	7.1 ± 6.9	10.6 ± 4.6	p=0.094
CD8+	9.8 ± 5.9	12.7 ± 5.3	p=0.115

Table 2. Correlations of percentage of the signed leucocytes from healthy population and Gaucher patients.

	Healthy control		Gaucher Disease patients	
	correlation	P value	correlation	P value
CD33+ leukocytes vs CD19+ leukocytes	0.560	0,013	0.205	0.571
CD33+ leukocytes vs CD14+ leukocytes	0.976	0,000	0.616	0.058
CD33+ leukocytes vs CD8+ leukocytes	0.746	0.000	0.036	0.922
CD19+ leukocytes vs CD14+ leukocytes	0.584	0,009	0.551	0.099
CD19+ leukocytes vs CD8+ leukocytes	0.792	0.000	0.506	0.136
CD14+ leukocytes vs CD8+ leukocytes	0.767	0.000	0.576	0.082

Discussion

We aimed to investigate the percentage of leukocytes having specific cell surface antigens (CD33, CD19, CD14, and CD8) and the glucocerebrosidase enzyme activity of these leukocytes in patients with GD. Our data indicated higher numbers of CD19+ leukocytes, and this may serve as a useful marker to predict leukocyte infiltration in GD patients.

Many authors [6,7, 13] have reported a higher prevalance of various types of cancer in GD patients; however, in a report from the International Gaucher Registry using data collected from their database including 2742 patients from more than 40 countries, the overall relative risk of cancer (breast, prostate, colon and rectum, lung, and hematologic malignancies other than myeloma) was 0.79 (95% CI: 0.67, 0.94), which does not reflect a statistically significant higher risk for GD patients. On the other hand, this study found that multiple myeloma incidence was 5- to 9-fold higher in GD patients than in the general population [8].

Various explanations have been proposed for the higher incidence of multiple myeloma in GD patients. One is related to the carcinogenic effect of sphingosine derived from glucosylsphingosine [14]; accumulated glycosylceramide becomes acylated to glucosylsphingosine, which might diffuse into the cytosol [6]. However, this hypothesis is unlikely because enzyme replacement therapy did not prevent lymphoma in these patients.

Another hypothesis is associated with the elevated levels of pro-inflammatory cytokines and chemokines through activation of macrophages by glucosylceramide accumulated within them [7, 15,16]. Chitotriosidase is a macrophage activation marker known to be higher in GD patients, and our data consistently showed higher chitotriosidase activity in these patients. Some investigations reported higher levels of cytokines IL-6 and CCL-18 in GD, which promote gammopathies [17, 18]. Cox et al.

pointed out that higher levels of cytokines, in particular, IL-6, might be responsible for chronic B cell stimulation and monoclonal gammopathy in GD [5].

Given that it has been presented that the plasma cells in B cell lymphomas express CD19 and/or CD4 [19], we determined the proportion of leukocytes expressing these antigens in GD patients. CD19 plays a major role in the B cell surface signal transduction complex and is expressed on mature B cells. Recently, it has been shown that there is a higher expression of CD19 in B cell malignancies such as acute lymphocytic leukemia (ALL), B cell non-Hodgkin lymphoma and chronic lymphocytic leukemia, but no expression in non-haematopoetic cells and other hematopoietic populations [9]. Pandey et al. [16] reported higher levels of chemokines and CD19+ leukocytes in the mouse model of GD. Marti et al. [3] showed high levels of CD19 leucocytes in 7/7 Gaucher patients, in accordance with our findings that is a higher number of CD19+ leukocytes in GD patients, which might predict hematologic malignancies. Recently, CD19 chimeric antigen receptor (CAR) directed T cells have shown very favorable clinical results in a range of B cell malignancies, even in patients with chemorefractory relapse [9]. Keeping in mind that CD19+ is an ideal CAR target, increased CD19+ expression in GD patients in our study may also provoke new clinical studies in which CAR therapy can be used in GD patients with B cell malignancies.

Our data showed no significant difference in CD8+, CD14+ and CD33+ leukocytes between GD patients and healthy controls, in accordance with the literature, which found a close association between these markers and the severity and/or prognosis of disease but not a diagnosis in multiple myeloma patients [20,21,22]. Because the occurrence of T cell lymphomas in a few patients with GD type 1 has been reported [20], we investigated the levels of CD8+ leukocytes (T-cytotoxic cells) and found them to be similar to those of healthy individuals. However, Lacerda et al. reported T cell deficiency as well as deficiency in CD8+ T cell subsets in GD patients, and this depletion was found to be related to sequestration in the spleen and improvement of bone pathology [23]. In our study, none of the patients had a splenectomy, 9 of them had a splenomegaly; therefore the percentage of CD8+ leucocytes remained into normal levels.

Shim et al. reported that immature and plasmablastic types of plasma cells were significantly associated with CD33 positivity [21], thus in our study, we also determined CD33 expressing leukocyte as a marker of the myeloid lineage in GD patients. Our data showed no significant difference in the proportion of CD33+ leukocytes in patients compared with controls, probably due to the absence of any type of lymphoma at the time of the study. We do not know if these patients will develop multiple myeloma in their lifetime, so we cannot conclude whether CD33+ might be a reliable marker for hematologic malignancies in the GD patient. As it has been shown that CD33 expression was an independent prognostic factor for poor prognosis in multiple myeloma [21], we can suggest that CD33 might be a prognostic rather than a predictive marker for further hematologic malignancy.

The main limitations of this study are both the study design (cross-sectional) and the limited number of patients. Nevertheless, since GD is a rare metabolic disease, we considered our results as an important initial data that deserves to be reported. We have found higher levels of CD19+ leukocytes in GD patients than in healthy controls. We conclude that higher levels of CD19 antigen expression supports the inflammatory activation hypothesis which might play a role in the development of B cell-derived malignancies. We suggest that higher CD19+ leukocyte levels may also serve as a marker to predict malignancy in these patients. To test this hypothesis, our group currently is further monitoring these patients for long-term survival and complications.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

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